Theriogenology 86 (2016) 1622–1629

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Establishment and characterization of buffalo fetal fibroblasts induced with human *telomerase reverse transcriptase*

Shun Zhang [a,b], Xiaomei Guan [c], Fenghua Lu [a], Jianrong Jiang [a], Yanfei Deng [a], Chan Luo [a], Deshun Shi [a,*]

[a] *Guangxi High Education Key Laboratory for Animal Reproduction and Biotechnology, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning, China*
[b] *Department of Reproductive Medical Center, The Affiliated Hospital of Guilin Medical University, Guilin, China*
[c] *Department of Reproductive Medical Center, People's Hospital of Luohu District, Shenzhen, China*

ARTICLE INFO

Article history:
Received 1 April 2016
Received in revised form 16 May 2016
Accepted 20 May 2016

Keywords:
Somatic cell nuclear transfer
Buffalo fetal fibroblast
hTERT
Lifespan
Donor cell

ABSTRACT

Fetal fibroblasts are often used as donor cells for SCNT, but their short lifespan greatly limits this application. To provide stable and long-lifespan cells, buffalo fetal fibroblasts (BFFs) transfected with human *telomerase reverse transcriptase* (*hTERT*). The *hTERT*-transfected BFFs (*hTERT*-BFFs) were evaluated by qRT-PCR, Western blot, karyotype analysis, telomerase activity assay, growth curve assay, flow cytometry, and soft agar assay. The development of SCNT embryos derived from *hTERT*-BFFs was also assessed *in vitro*. The morphology of *hTERT*-BFFs was similar to the nontransfected BFFs, and the karyotype of *hTERT*-BFFs was normal at passage 30. The *hTERT*-BFFs at passage 4 and 30 had higher telomerase activity and extended proliferative lifespan with an increase in cell population at S phase when compared with nontransfected BFFs at passage 5 and 30. The mRNA expression of *p53* in *hTERT*-BFFs at passage 5 and 30 remained unchanged when compared with nontransfected BFFs at passage 5, whereas the mRNA expression of *p53* in the nontransfected BFFs at passage 30 was increased. Soft agar assay showed that *hTERT*-BFFs at passage 30 were not a malignant phenotype. Significantly, more SCNT embryos derived from *hTERT*-BFFs at passage 5 and 30 developed to blastocysts in comparison with BFFs at passage 30. The *Caudal type homeobox 2* and *Connexin 43* genes were indicated to involve in the development of cloned embryos. These results indicate that transfection of BFFs with *hTERT* can extend their lifespan and retain their basic and key biological characteristics in the status of primary BFFs.

1. Introduction

Somatic cell nuclear transfer has been applied in mammals as a valuable tool in embryological studies and a method for generation of transgenic large domestic animals [1–3]. Fetal fibroblast cells are often used as donor cells because of easy culturing and genetic manipulation. However, with increasing passages of donor cells, the blastocyst development of SCNT embryos decreases because of the limited lifespan of donor cells [4–6]. Therefore, enhancing the lifespan of donor cells *in vitro* may be a new strategy to overcome the critical barrier for SCNT.

Telomerase is a ribonucleo-protein enzyme complex that adds repeat telomeric sequences to the ends of chromosomes, which counteracts the shortening of telomeres [7].

Shun Zhang, Xiaomei Guan and Fenghua Lu contributed equally to this work.

* Corresponding author. Tel.: +86 771 3239202; fax: +86 771 3239202.
E-mail address: ardsshi@gxu.edu.cn (D. Shi).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.05.023

Telomeres progressively shorten in almost all dividing cells as most somatic cells do not maintain sufficient telomerase activity [8]. Human *telomerase reverse transcriptase* (*hTERT*) is the limiting factor for maintaining telomerase activity in most somatic cells, and its expression is the most common means by which cells acquire replicative immortality [9]. Immortalized mammary fibroblasts [10], gingival fibroblasts [11], goat mammary epithelial cells [12], and human amniotic epithelial cells [13] were successfully established by inducing *hTERT* expression in them.

Buffalo is an important economic domestic animal in China. To date, the production of transgenic buffalo is far behind other domestic animals largely due to the limited availability of appropriate donor cells. In the present study, buffalo fetal fibroblasts (BFFs) were transfected with *hTERT*. Then, the biological characteristics of transfected BFFs, including expression of *hTERT*, telomerase activity, proliferative ability, cell cycle, and *p53* expression as well as tumorigenicity were investigated. Finally, the development of SCNT embryos derived from hTERT-BFFs was further examined.

2. Materials and methods

2.1. Cell isolation and culture

Buffalo fetus was obtained from a 40-days pregnant female buffalo under sterile condition. Fetal skin tissue samples were cut into 2-mm pieces and then cultured in DMEM (GIBCO, Rockville, USA) containing 10% fetal bovine serum (FBS; GIBCO). Fibroblast cells have been purified after two to three times of subculture for 8 hours, based on the different resistance, adherence property, and cell activity of epithelioid cells and fibroblast cells. The culture medium was changed every second day.

The cDNA sequence of the catalytic subunit of *hTERT* was cut out of pMXS-hTERT plasmid with EcoRI and NotI and inserted into pMXs-IRES-Neo plasmid (Fig. S1). When BBF cells at passage 5 reached 60% confluence, cells were infected with packed virus in the presence of polybrene (6 μg/mL; Sigma, St. Louis, USA). Fourty-eight hours after transfection, the transfection mixture was replaced with DMEM medium with 10% FBS. Cells carrying the target plasmid were selected by 800 μg/mL G418 (Sigma). When all the uninfected cells are dead, we halved the G418 concentration and kept screening for positive cell clones. Segmental digestion process was used to obtain positive cell clones.

For morphological analysis, cells were cultured in DMEM medium with 10% FBS in cell culture grade petri dishes containing an uncoated cover slide. When cells reached about 80% confluence, phase-contrast images of cells were taken by AxioCamHm camera (Carl Zeiss, Inc., New York, USA).

2.2. Karyotype analysis

Chromosomes were analyzed from actively proliferating cultures of *hTERT*-BFFs at passage 30. Positive cell clones were treated with colchicine at 37 °C for 2 hours, trypsinized, centrifuged, and then incubated in 0.05-M KCl at 37 °C for 20 minutes. Cells subsequently were fixed with ice-cold acetic acid/methanol (1:3, v/v) and collected by centrifugation. Thereafter, the dispersed cell suspension was smeared on a cold slide, air dried, stained with Giemsa (Sigma), and observed microscopically.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNAs were isolated from cells or cloned embryos using Trizol reagent (Invitrogen, Waltham, MA, USA). The TaqMan Reverse Transcription Kit (Takara, Dalian, China) was used to obtain cDNA for mRNA detection. For *hTERT, p53, Caudal type homeobox 2 (Cdx2)* and *Connexin 43 (Cx43)* mRNA, qRT-PCR was performed using SYBR Green PCR Kit (Takara) according to the manufacturer's instructions, respectively. *GAPDH* were used as internal controls. The primers for *hTERT* mRNA are: forward, 5′-TTCHHCGACTG-CAAGCTC -3′ and reverse, 5′-CACAGCCTCAGCATCTTCAATG -3′; the primers for *p53* mRNA are: forward, 5′- TCTGGGA-CAGCCAAGTCTGTG -3′ and reverse, 5′- TTTCCTTCCACTCG-GATAAGATG -3′; the primers for *Cdx2* mRNA are: forward, 5′- GCAAAGGAAAGGAAAATCAACAA -3′ and reverse, 5′-GGGCTCTGGGACGCTTCT -3'; the primers for *Cx43* mRNA are: forward, 5′- TATGCTTATTTCAATGGCTG -3′ and reverse, 5′-AGTTAGAGATGGTGCTGCCT -3'. Data were expressed as fold changes relative to *GAPDH* calculated based on the following formula: $RQ = 2^{-\triangle\triangle Ct}$.

2.4. Western blot

Cells were lysed in sodium dodecyl sulfate lysis buffer containing protease inhibitor, and the protein concentration was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer's instructions. Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene fluoride membrane. The following primary antibodies were used: rabbit anti-hTERT (1:1000; Epitomics, Burlingame, USA) and mouse anti-β-actin (1:5000; Cell Signaling Technology, Boston, USA). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies (1:4000; Abcam, Cambridge, USA). The membranes were exposed using a ChemoDoc XRS detection system (Bio-Rad, Milan, Italy).

2.5. Telomerase activity assay

Telomerase activity was assayed by using TRAPEZE Telomerase Detection Kit S7700 (ChemiconInternatinal Inc., Temecula, USA), according to the manufacturer's instructions. TSR8 is used as positive control and 85 °C 10 minutes heat-inactivated samples were used as negative controls. Cell extracts were separately prepared by CHAPS lysis buffer and amplified by PCR. The PCR products were separated in a 12.5% nondenaturing PAGE. Gels were stained with ethidium bromide and photographed by a ChemoDoc XRS detection system (Bio-Rad, Milan, Italy).

2.6. Growth curve assays

The hTERT-BFFs at passage 5 and Passage 30 and nontransfected BFFs at passage 5 and 30 were seeded in 24-well plates at 1×10^4 cells/well. In each day of 9 days of